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All of the mouse experiments were performed by Shawn Skerrett, Harborview Medical Center, Seattle, except for the *Influenza virus* experiments which were performed by John Kash and Jeffrey Taubenberger, at the NIH. The primate studies were performed by Luis de Silva and Michael Ingram, at USAMRIID.

14. ABSTRACT
The overall objective for this project was to discover and validate host molecular fingerprints that classify inhaled microbial (bacterial and viral) biothreat agents affecting the lung. Aerosol infection experiments were performed in mice and primates, and host biomarkers were identified in lung tissue and peripheral blood plasma that classify the identity of the infectious agents. Through extensive experiments in mice, biomarkers were identified that distinguish between different bacterial biothreat agents, between virulent and less-virulent strains of the same bacterial agents, between biothreat and non-biothreat lung pathogenic agents, and that distinguish between bacterial and viral biothreat agents. Biomarkers were identified that classify the biothreat agents during the pre-symptomatic phase of the infection time course, and that are consistently useful when sampled at any of the time points throughout the time course of the infection. Blood biomarkers were identified in the mouse model of infection that also were useful as blood biomarkers in limited studies performed in two primate models of biothreat infections.

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Executive Summary

The overall objective for this project was to discover and validate host molecular fingerprints that classify inhaled microbial (bacterial and viral) biothreat agents affecting the lung. Aerosol infection experiments were performed in mice and primates, and host biomarkers were identified in lung tissue and peripheral blood plasma that classify the identity of the infectious agents. Through extensive experiments in mice, biomarkers were identified that distinguish between different bacterial biothreat agents, between virulent and less-virulent strains of the same bacterial agents, between biothreat and non-biothreat lung pathogenic agents, and that distinguish between bacterial and viral biothreat agents. Biomarkers were identified that classify the biothreat agents during the pre-symptomatic phase of the infection time course, and that are consistently useful when sampled at any of the time points throughout the time course of the infection. Blood biomarkers were identified in the mouse model of infection that also were useful as blood biomarkers in limited studies performed in two primate models of biothreat infections.

1. Scientific Summary

We established collaborations to be able to perform inhaled biothreat exposure experiments in mice and primates. All of the mouse experiments were performed by Shawn Skerrett, Harborview Medical Center, Seattle, except for the *Influenza virus* experiments which were performed by John Kash and Jeffrey Taubenberger, at the NIH. The primate studies were performed by Luis de Silva and Michael Ingram, at USAMRIID.

Experiments were performed in three strains of mice (BALB/c, C57BL/6, Swiss Webster) with the virulent and the matched less-virulent strains of the biothreat agents: *Francisella tularensis* SchuS4, *Francisella tularensis* subspecies *holarctica* live vaccine strain (LVS), *Francisella tularensis* subspecies *novicida*, *Yersinia pestis* CO92, *Yersinia pestis* CO92 pYV- (virulence plasmid-deficient). Biothreat viral pathogens included the pandemic 1918 H1N1 influenza virus. Non-biothreat lung pathogens were also studied, including *Pseudomonas aeruginosa* PAK, *Legionella pneumophila* Philadelphia-1, and H1N1 *Influenza* virus (2009 pandemic Mex09, seasonal NIH50) (**Task 1**).

The biothreat agents were aerosolized and delivered by inhalation. The inoculum dose was chosen to be the lowest reproducible dose of virulent strain that caused 100% mortality at day 5. The virulent *Francisella tularensis* and *Yersinia pestis* experiments resulted in disseminated, uncontrolled infections with little or no evidence of a host innate immune response in the first 24h. For the matched less-virulent strain, we chose a dose of agent that was orders of magnitude higher, and that induced cellular infiltrates into the lung and activated measurable markers of inflammation in bronchoalveolar fluid, though due to the attenuated virulence, did not cause mortality. For *Francisella tularensis*, ~150cfu/lung were deposited for the virulent bacterial strain and ~10^4 CFU/lung were deposited for the less-virulent strains. For *Yersinia pestis*, 300-600 CFU/lung were deposited in C57BL/6 and BALB/c mice, and ~3000 CFU/lung in Swiss Webster mice. For the viral select agent, 1918 influenza virus, 1000 PFU was administered intranasally which results in 100% mortality by day 5-7 post-exposure. For the less-virulent *Yersinia pestis* CO92 pYV$^-$ strain, 5000 CFU/lung were deposited and caused mild lung inflammation and clearance of organisms without mortality. For the less virulent influenza H1N1 strains (NIH50/Mex09), inoculation of 10^5 PFU results in significant weight loss (10-20%) but 100% survival.

Pseudomonas aeruginosa experiments were performed with a deposition of ~5 x 10^5 CFU/lung, and *Legionella pneumophila* experiments were performed with a deposition of ~1 x 10^6 CFU/lung. Infections with these non-biothreat lung pathogens resulted in vigorous host responses and successful pathogen clearance. Some differences among the three strains of mice were noted but overall the patterns of response were similar.

Samples were collected at 4h, 24h, and 48h after exposure. We focused on lung tissue and blood, though many organs were collected and banked. For each of these tissues, mRNA, miRNA and protein were measured and analyzed (**Tasks 2, 3, 4, 5).**

a. microRNA profiling

During the course of executing this contract, we developed the capacity to measure the abundance of microRNAs (miRNAs) in blood plasma. miRNAs are non-coding regulatory RNAs with a length of 19-23 nucleotides. In a separate project studying drug-induced liver injury, we determined that circulating miRNA (such as the liver specific miR-122) is a useful marker of liver damage. We thus conducted extensive miRNA

profiling studies in the tissue and blood plasma samples collected from biothreat agent exposure experiments, and have determined which miRNAs are useful biomarkers to classify infections. We have identified that: 1) plasma mmu-miR-494 distinguishes between virulent and less-virulent *Francisella tularensis*, in all 3 strains of mice; 2) plasma mmu-miR-30e distinguishes between less-virulent *Francisella tularensis* and less-virulent *Yersinia*, in all 3 strains of mice; 3) miR-223, miR-125a-5p, miR-192 and miR-155 distinguish between *Francisella* and *Yersinia* infections, in all three strains of mice, at all time points; 4) miR-322 in lung tissue distinguishes between virulent and avirulent *Yersinia*, in all three strains of mice, and at all time points; and 5) multiple miRNAs in plasma distinguish between biothreat and non-biothreat lung pathogenic agents (miR-106b, miR-21, miR-25, miR-30a, miR-93, miR-20a, miR-451, miR-19b, let-7i, miR-532-5p, miR-218, miR-152, miR-27b, miR-200, miR-127, miR-337-5p, miR-362-3p, miR-409-3p, miR-18a, miR-652, miR-324-5p, miR-532-3p).

Interestingly, we note several instances where the miRNA measured in blood plasma is more informative than the form measured in tissue (including examples 1 and 2, above). In addition, we have constructed a database from our sequencing data that catalogs the entire repertoire of miRNA sequences (http://galas.systemsbiology.net/cgi-bin/isomir/find.pl), which enables users to determine the most abundant sequence and the degree of heterogeneity for each individual miRNA species. This information will be useful both to better understand the functions of isomirs and to improve probe or primer design for miRNA detection and measurement (Lee LW, et al 2010).

b. Proteomics

We have used a variety of proteomic strategies to discover protein biomarkers of infection in lung tissue, bronchoalveolar fluid, and in blood plasma. Overall, in this contract, the "shotgun" mass spectrometry approach has not been useful, as it is dominated by the repeated detection of very high abundance proteins that typically are not informative biomarkers for agent classification or for early pre-symptomatic detection of biothreat infections.

The "targeted" proteomic strategy of selective reaction monitoring (SRM) holds greater promise, as sets of pre-defined proteins can be quantified with greater sensitivity than

"shotgun" approaches. During the course of this contract, we have developed a targeted proteomics strategy termed "index-ion triggered MS2 ion quantification" (iMSTIQ) that allows reproducible and accurate peptide quantification in complex mixtures (Yan, et. al., MCP, 2011). The key feature of iMSTIQ is that using index-ion triggered analysis, the acquisition of full MS2 spectra of targeted peptides is enabled independent of their ion intensities. Accurate quantification is achieved by comparing the relative intensities of multiple pairs of fragment ions derived from isobaric targeted peptides during MS2 analysis. The iMSTIQ approach has not yielded informative biomarkers within this contract, though its development is an exciting prospect for future studies.

c. Mouse and primate mRNA gene expression profiling to classify infectious (Task 6, 7, 8, 9, 10, 11, 12)

We have performed an extensive series of time-course infection experiments in mice (BALB/c, C57BL/6, Swiss Webster) for a panel of respiratory pathogens, including exposures with both virulent and avirulent strains of bacterial biothreat agents (*Yersinia pestis, Francisella tularensis*), viral biothreat agent (1918 pandemic *Influenza virus*), as well as non-biothreat agents (*Legionella pneumophila, Pseudomonas aeruginosa,*). Through collaborative efforts with researchers at USAMRIID we have also received a limited number of samples from non-human primates exposed to either virulent *Yersinia pestis* (African green monkey) or *Francisella tularensis* SchuS4 (*Rhesus macaque*).

Analysis of mRNA expression profiles of lung tissue from infected mice has led to identification of signatures that distinguish *Y. pestis* and *F. tularensis* (including Csf1r, Fn1, Mmp14, Lrp6, full list in Figure 1, HDTRA 1-08-C-0023 No Cost Extension Period Q1 4.2 technical attachment), as well as virulent and avirulent *F. tularensis* (including Bmpr2, Lrp5, Ptpn13, Ptprf, Vegfa, full list in Figure 2) and *Y. pestis* infection (IL4ra, Cflar, and Figure 3). We have also identified signatures capable of distinguishing bacterial and viral select agents (Tac2, Selp, Il6ra, Lrp6, Rock1, and Figure 4). Importantly, these signatures are time-independent and are detectable as early as 4hrs post-exposure. Finally, using a Ranking analysis performed in GeneData's Analyst software, we identified both the optimal gene set and the prediction algorithm that most accurately classifies all respiratory pathogens, including those that induce a non-lethal but

severe infection (*L. pneumophila* and *P. aeruginosa*). For this analysis, data from the 4hr post-exposure was used as the training set which resulted in an estimated prediction error rate of < 2% with a minimum gene set of approximately 1400 sequences (See Figure 5A). Subsequent classification of the 24 and 48hr post-exposure datasets using these criteria resulted in 100% accuracy (data not shown) for all pathogens. Importantly, although these are lung-based mRNA signatures, many of these sequences could potentially be detectable in peripheral blood, either because they are expressed in immune cells and/or encode secreted proteins (Figure 5B). Each pathogen is associated with a distinct pattern of expression of these sequences which collectively enables accurate identification. For example, ICAM1 is strongly induced during infection with non-lethal infection, slightly induced by 1918 influenza virus, and suppressed by bacterial select agents, Fn1 is uniquely induced by 1918 influenza virus, Bmpr2, Clip1, Cast, Mgea6 are uniquely induced by *F. tularensis*, many chemokines and immune-cell specific markers (including CCL20, CXCL10, CCL2, CCL4, CCL17, TNF, CD14, IL1RN) are induced by non-lethal infections at all time-points while suppressed during early infection with all select agents.

Collectively, the results of these studies demonstrate that characterizing the host response to infection can ultimately lead to the identification of biomarkers capable of accurately classifying a wide range of respiratory pathogens at multiple time-points post-exposure. Importantly, these signatures are all detected as early as 4hrs post-exposure at which time there is minimal pathogen replication, little to no histopathological changes in lung tissue and animals show no obvious signs of illness. The ability to both detect and accurately classify bio-threat agents during the pre-symptomatic phase of infection is critical to facilitate appropriate therapeutic intervention and surveillance.

2. Collaborations

- Lucy Carruth, Applied Physics Laboratory, Johns Hopkins University
- Jeffery Taubenberger, NIAID, NIH

3. Publications

1. Wang, K., Lee, I. Y., Hood, L., and Galas, D. J. (2010) Systems Biology and the Discovery of Diagnostic Biomarkers. *Disease Markers* 28 199-207.

2. Weber, J., Baxter, D., Zhang, S., Huang, K. H., Lee, M. J., Galas, D. J. and Wang, K. (2010) The microRNA spectrum in 12 body fluids. *Clin Chem.* 56:1733-41.
3. Lee, L. W., Etheridge, A., Zhang, S., Ma, L., Martin, D., Galas, D. J., and Wang, K. (2010) Complexity of the microRNA repertoire revealed by next-generation sequencing. *RNA*. 16:2170-80.
4. Vêncio, E. F., Pascal, L. E., Page, L. S., Gareth Denyer, G., Wang, A. J., Ruohola-Baker, H., Zhang, S., Wang, K., Galas, D. J., and Liu, A. Y. (2011) Embryonal carcinoma cell induction of miRNA and mRNA changes in co-cultured prostate stromal fibromuscular cells. *J Cell Physiol.* 226: 1479-1488.
5. Cho, J. H., Wang, K., and Galas, D. J. (2011) An integrative approach to construct biologically meaningful modules. *BMC Systems Biology*, 26:117-126.
6. Kash, J.C., Walters, K. -A., Davis, A. S., Sandouk, A., Schwartzman, L. M., Jagger, B. W., Chertow, D. S., Li, W., Kuestner, R. E., Ozinsky, A., and Taubenberger, J. K. Lethal synergism of 2009 pandemic H1N1 Influenza virus and Streptococcus pneumoniae coinfection is associated with loss of murine lung repair responses. *MBio*, 2011 September/October: 2(5): e00172-11.
7. Etheridge, A., Lee., I. Y., Hood, L. E., Galas, D. J., and Wang, K. (2011) Extracellular microRNA: a new source of biomarkers. *Mutation Res.* 717:85-90.
8. Lee, M. J., Gho, J. H., Galas, D. J., and Wang, K., (2012) The systems biology of neurofibromatosis type 1 – Critical roles for microRNA. *Exp Neurol.* In press.
9. Wang, K., Yuan, Y., Cho, J. H., Baxter, D., and Galas, D. J. (2012) Systematic comparison of the microRNA spectrum between serum and plasma. Submitted to *PLoS One*.
10. Jagger, B. W., Wise, H. M., Kash, J.C., Walters, K.-A., Wills, N. M., Dunfee, R. L., Schwartzman, L. M., Ozinsky, A., Bell, G. L., Dalton, R. M., Lol, A., Efstathiou, S., Atkins, J.F., Firth, A.E., Taubenberger, J.K., and Digard, P., A. (2012) Novel Influenza A Virus Protein Encoded by an Overlapping Reading Frame in Segment 3 Modulates the Host Response. Submitted to *Science*.
11. John C. Kash, Yongli Xiao, A. Sally Davis, Kathie-Anne Walters, Daniel S. Chertow, Judith D. Easterbrook, Rebecca L. Dunfee, Aline Sandouk, Louis M. Schwartzman, Nancy Wehr, Adrian Ozinsky, Rodney L. Levine, Susan Doctrow

and Jeffery K. Taubenberger. (2012) A catalytic scavenger of reactive oxygen species reduces lung damage and increases survival during 1918 influenza virus infection. Submitted to *Cell Host & Microbe*.

4. **Presentations (Kathie A. Walters)**
   - Kathie-Anne Walters, John C. Kash, R.E Kuestner, A. S. Davis, J.K. Taubenberger, A. Ozinsky, Pandemic 2009 H1N1 determines outcome of Streptococcus pneumonia co-infection. Cell Symposia: Influenza : Translating basic insights, Washington, DC, 2010
   - Kathie-Anne Walters, Shawn Skerrett, Kai Wang, Rachael Anne Olsufka, Candice Suping Huang, Rolf Kuestner, Bruz Marzolf, Leroy Hood, Adrian Ozinsky. Molecular Signatures that Classify the Host Response to Inhaled Lung Infection. ASM Biodefense Meeting, Baltimore, MD, 2010

5. **Invited Presentations (Kai Wang)**
   - 49th Annual Meeting of the Society of Toxicology, 2010, Salt Lake City, UT
   - Use of microRNA in toxicological application workshop, 2010, hosted by ILSI health and environmental science institute. Arlington, VA
   - BIT's Tetra-Congress of MolMed-2010, Session Chair, Shanghai, China
   - microRNA in Human Disease & Development, Cambridge Healthtech Institute, 2011 Boston, MA
   - Annual convention of the American Association of Pharmaceutical Scientists, 2011 Washington DC.
   - Annual convention of the American Society of Nephrology 2011 Philadelphia, PA.
   - Southern California SOT Regional Chapter annual meeting 2011, Los Angeles, CA.
   - US HUPO annual meeting 2012 San Francisco, CA
   - 51th Annual Meeting of the Society of Toxicology, 2012, San Francisco, CA